Meiotic and mitotic aneuploidies drive arrest of in vitro fertilized human preimplantation embryos

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Abstract

The high incidence of chromosome aneuploidy in early human development, arising either from segregation errors in meiosis or postzygotic mitosis, is the primary cause of pregnancy loss, miscarriage, and still birth following natural conception as well as *in vitro* fertilization (IVF). Preimplantation genetic testing for aneuploidy within trophectoderm biopsies of IVF embryos at the blastocyst stage has confirmed the prevalence of aneuploidies of putative meiotic and postzygotic mitotic origins. However, only about half of normally fertilized embryos develop to the blastocyst stage *in vitro*, while the others arrest at cleavage to late morula or early blastocyst stages. To investigate the role of aneuploidy in developmental arrest, we compared the incidence of various forms of aneuploidy in a large series (n = 909) of trophectoderm biopsies and arrested embryos and correlated them with abnormalities of the first two cleavage divisions using time lapse imaging (n = 843). The combined incidence of meiotic and mitotic aneuploidies was strongly associated with blastocyst morphological grading, with the proportion ranging from 20% to 90% for the highest to lowest grades, respectively. In contrast, the incidence of aneuploidy among arrested embryos was exceptionally high (94%), dominated by mitotic aneuploidies affecting multiple chromosomes. In turn, these mitotic aneuploidies were strongly associated with abnormal cleavage divisions, such that 51% of abnormally dividing embryos possessed mitotic aneuploidies compared to only 23% of normally dividing embryos. We conclude that the combination of meiotic and mitotic aneuploidies, the latter possibly exacerbated by suboptimal culture conditions, drives arrest of human embryos *in vitro*, as development increasingly relies on embryonic gene expression at the blastocyst stage. We discuss the implications of these findings for both basic human developmental biology and clinical IVF.

Introduction

Following natural conception, many human embryos are chromosomally abnormal and are progressively eliminated through preclinical pregnancy loss, miscarriage, and still birth, such that the overall incidence of these abnormalities detected in newborns is less than 1% (Gardner and Amor, 2018; Moorthie et al., 2018). Observed chromosome abnormalities include genome-wide abnormalities in ploidy (e.g., triploidy), as well as whole and segmental aneuploidies of individual chromosomes (Levy et al., 2014). Whole chromosome aneuploidy frequently arises through errors in meiosis, predominantly in females, resulting in aneuploid oocytes. Risk of such maternal meiotic aneuploidies, particularly of the smaller and acrocentric chromosomes, increases exponentially for women over the age of 35 years in parallel with increasing risk of miscarriage (Nagaoka et al., 2012). A similar pattern of aneuploidy is also observed following *in vitro* fertilization (IVF) (Segawa et al., 2017; Ishihara et al., 2020). Hence, preimplantation genetic testing for aneuploidy (PGT-A) at the blastocyst stage, by trophectoderm biopsy and next-generation sequencing (NGS)-based chromosome copy number analysis, is now widely used for the selection of viable euploid blastocysts for transfer (Rosenwaks and Handyside, 2018).

Chromosome abnormalities identified by PGT-A at the blastocyst stage include whole or segmental, full or intermediate chromosome copy number changes (McCoy, 2017). Whereas meiotic errors produce abnormalities that uniformly affect all cells of the embryo and therefore result in full (i.e., integer) signatures of trisomies or monosomies in the biopsied trophectoderm cells, mitotic errors result in chromosome mosaicism, defined by multiple cell lineages with distinct chromosome complements and manifest as intermediate (i.e., fractional) copy number changes due to averaging across cells. Chromosome missegregation is common during the early mitotic cleavage divisions and is frequently accompanied by abnormalities of cell division. These include cellular fragmentation and uneven cleavage resulting in anuclear fragments, various forms of multinucleation, as well as tripolar or other spindle abnormalities, which have also been observed in the trophectoderm at the blastocyst stage (Chatzimeletiou et al., 2005). For example, errors can occur at syngamy causing the loss of multiple chromosomes from one parent (So et al., 2022), and tripolar divisions in early cleavage result in sub-diploid clones of cells which subsequently lead to developmental arrest prior to the blastocyst stage (Ottolini et al., 2017; McCollin et al., 2020).

Previous comparisons of single cells biopsied from cleavage-stage embryos and multiple trophectoderm cells biopsied from blastocysts demonstrated a reduced incidence of certain forms of chromosome abnormalities at the blastocyst stage (McCoy et al., 2015a, b; McCoy et al., 2018). These include mitotic aneuploidies and karyotype-wide ploidy abnormalities presumably caused by abnormal fertilization. However, only

about half of normally fertilized embryos reach the blastocyst stage, while the remainder arrest at various cleavage, late morula, or early blastocyst stages (Biggers and Summers, 2008; Summers et al., 2013; Sfontouris et al., 2016; Werner et al., 2016). Thus, the relationship between errors in cell division, the resulting chromosome abnormalities, and developmental arrest remains poorly understood.

To investigate the role of aneuploidy in developmental arrest, we compared the incidence of various forms of aneuploidy in a large series (n = 909) of trophectoderm biopsies and arrested embryos and correlated them with abnormalities of the first two cleavage divisions using time-lapse imaging (n = 843). Using copy number signatures to distinguish aneuploidies of meiotic versus mitotic origin, we demonstrate that both forms of aneuploidy are reduced in developing blastocysts compared to arrested embryos, consistent with their contributions to early embryonic mortality. Using statistical modeling, we further show that the origin (meiotic versus mitotic) and number of aneuploid chromosomes affecting a given embryo are strongly associated with the probability and timing of embryonic arrest following IVF, as well as the morphological grade of embryos that survive to blastocysts. Together, our study offers a detailed and expansive view of the impacts of diverse chromosome abnormalities on early human embryo development, while also lending insight into the reasons why many embryos derived from euploid zygotes ultimately arrest. In addition to the broad implications for basic understanding of human preimplantation development, our findings help clarify the factors that impact cumulative pregnancy rates following clinical IVF.

Results

Between January 2016 and June 2018, 125 patients (mean 38.9 years at oocyte retrieval; range 30-45 years) underwent a total of 165 IVF cycles with extended embryo culture to the blastocyst stage and biopsy of 5-10 trophectoderm cells on days 5-7 post-insemination for PGT-A. Excluding an additional 22 cycles in which all embryos arrested and were not tested by PGT-A (a total of 52 zygotes with two pronuclei [2PN]), an average of 3.8 of 7.2 (53%) embryos reached the blastocyst stage per cycle—a proportion that declined with maternal age (Quasibinomial GLM: $\hat{\beta}$ = -0.080, SE = 0.022, $p = 3.7 \times 10^{-4}$; Supplementary Table 2). Considering all 1232 normally fertilized (2PN) zygotes and excluding possible triploid embryos subsequently identified by PGT-A (see Methods), 622 (50.5%) embryos developed to the blastocyst stage and 610 (49.5%) embryos arrested between the zygote and late morula/early blastocyst stages (Figure 1). A total of 909 (73.4%) embryos derived from 2PN zygotes were tested with PGT-A, including 612 of the 622 (98.4%) blastocysts and 297 of the 610 (48.7%) arrested

embryos. We highlight the investigation of arrested embryos as well as poorer quality blastocysts (257 embryos with morphological grades BC, CC, CD, DC, or DD) as important aspects of our study, as such embryos are not as frequently tested in clinical settings because they are not typically considered as good candidates for biopsy, vitrification, or transfer.

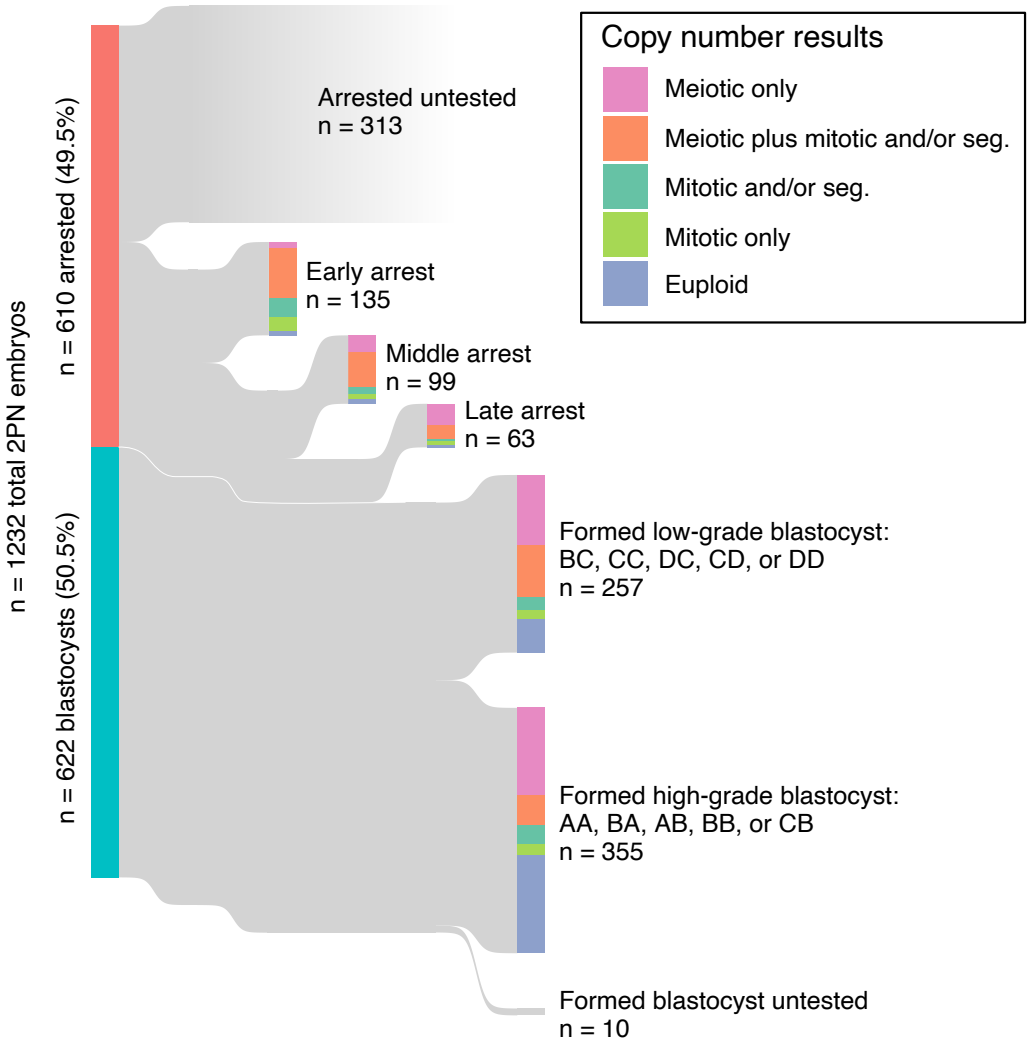

Figure 1. Developmental outcomes of 1232 normally fertilized (2PN) embryos and their associated PGT-A results. A total of 909 (73.8%) of embryos were tested with PGT-A, including 297 of 610 (48.7%) arrested embryos and 612 of 622 (98.4%) blastocysts.

Meiotic and mitotic aneuploidies are prevalent among preimplantation embryos

Across 909 tested embryos, 206 (22.6%) were euploid while 703 (77.3%) possessed whole or segmental aneuploidies of one or more chromosomes. To gain insight into the origins of aneuploidies and their consequences for development, we distinguished putative meiotic and mitotic aneuploidies based on their PGT-A copy number profiles (see Methods). Along with 50 aneuploidies of entire sex chromosomes, for which discerning mosaic aneuploidy poses unique challenges (see Methods), we discovered 1154 putative meiotic and 1051 putative mitotic aneuploidies of whole autosomes. We additionally identified 358 large segmental aneuploidies (on the scale of entire chromosome arms), including 351 affecting autosomes and 7 affecting sex chromosomes. Consistent with previous work (e.g., McCoy et al. 2015b), the meiotic aneuploidies disproportionately impacted chromosomes 15, 16, 19, 21, and 22, whereas mitotic aneuploidies exhibited similar frequencies across all autosomes (Figure 2A). Moreover, we confirmed that the putative meiotic aneuploidies possessed a strong association with maternal age (Quasibinomial generalized linear model [GLM]: $\hat{\beta}$ = 0.261, standard error [SE] = 0.032, *p* value [*p*] = 1.68×10^{-12}), whereas putative mitotic aneuploidies exhibited no significant age association (Quasibinomial GLM: $\hat{\beta}$ = 0.009, SE = 0.033, *p* = 0.799).

We observed no significant co-occurrence of meiotic and mitotic aneuploidies affecting different chromosomes of the same embryos (Fisher's Exact Test: odds ratio [OR] = 1.32, 95% confidence interval [CI; 0.99, 1.77], *p* = 0.055), suggesting that these error mechanisms are largely independent. However, when restricting to the 612 blastocyst-stage embryos, we observe a slight enrichment of mitotic aneuploidy among embryos already affected by meiotic aneuploidy (Fisher's Exact Test: OR = 1.54, 95% CI [1.02, 2.36], *p* = 0.036). While the wide confidence intervals suggest that our power for detecting such an effect is limited in both cases, the latter observation raises the intriguing hypothesis that following embryonic genome activation, the functional effects of meiotic aneuploidies may include impacts on the mitotic machinery, compromising the fidelity of chromosome segregation.

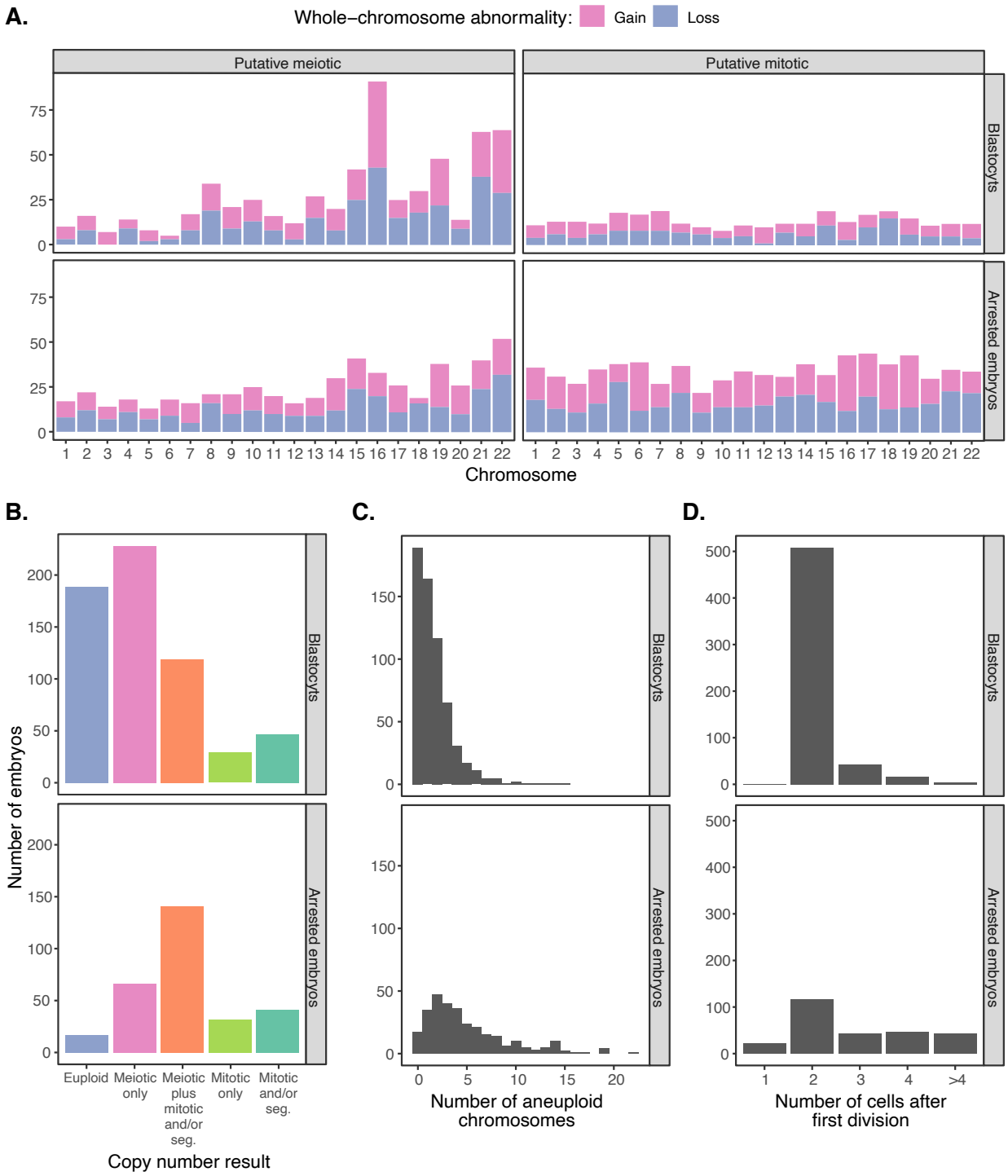

Figure 2. Characteristics of blastocysts and arrested embryos. **A.** Chromosome-specific counts of putative meiotic (i.e., full copy number change) and putative mitotic (i.e., intermediate copy number change) whole-chromosome gains and losses observed in blastocysts and arrested embryos as determined with PGT-A. Only autosomes are depicted, as distinguishing meiotic and mitotic origins of aneuploidies affecting sex chromosomes poses unique challenges (see

Methods). **B.** Counts of arrested embryos versus developing blastocysts, stratified by PGT-A copy number result category. **C.** Counts of arrested embryos versus developing blastocysts, stratified by total number of aneuploid chromosomes. **D.** Counts of arrested embryos versus developing blastocysts, stratified by number of cells present after the first mitotic division.

Mitotic aneuploidies disproportionately contribute to preimplantation arrest

The overall incidence of aneuploidy among arrested embryos was 94%, compared to 69% in tested blastocysts. Both putative meiotic and putative mitotic whole-chromosome aneuploidies were enriched among arrested embryos compared to embryos that developed to the blastocyst stage, though the effect was much stronger for mitotic aneuploidies (Fisher's Exact Test: OR = 6.02, 95% CI [4.40, 8.28], $p = 4.22 \times 10^{-33}$) compared to meiotic aneuploidies (Fisher's Exact Test: OR = 1.62, 95% CI [1.20, 2.20], $p = 0.0012$).

For the purpose of downstream analysis and visualization, we further grouped embryos into one of five mutually exclusive categories: (1) euploid, (2) meiotic whole-chromosome aneuploidy only, (3) meiotic whole-chromosome aneuploidy in combination with mitotic whole-chromosome aneuploidy or segmental aneuploidy (4) mitotic whole-chromosome aneuploidy only, and (5) segmental aneuploidy with or without mitotic whole-chromosome aneuploidy (Table 1). The distribution of embryos among these categories was significantly different for arrested embryos compared to trophectoderm biopsies of developing blastocysts (Figure 2B; Pearson's Chi-squared Test: X^2 [4, N = 909] = 143.4, $p = 5.41 \times 10^{-30}$).

Putative chromosome status	Abbreviation	Whole arrested embryos	Blastocyst-stage embryos (trophectoderm biopsies)
Euploid	Euploid	17 (6%)	189 (31%)
Meiotic whole-chromosome aneuploidy only	Meiotic only	66 (22%)	228 (37%)
Meiotic whole-chromosome aneuploidy with mitotic whole-chromosome aneuploidy or segmental aneuploidy	Meiotic plus mitotic and/or seg.	141 (47%)	119 (19%)
Mitotic whole-chromosome aneuploidy only	Mitotic only	32 (11%)	29 (5%)
Segmental aneuploidy with or without mitotic whole-chromosome aneuploidy	Mitotic and/or seg.	41 (14%)	47 (8%)
Total		297	612

Table 1. Incidence of various forms of aneuploidy in whole arrested embryos (see Methods for description of arrested embryos) versus trophectoderm biopsies of developing blastocysts as

determined by preimplantation genetic testing for aneuploidy (PGT-A). Our approach for distinguishing putative meiotic and mitotic aneuploidies based on chromosome copy number results is detailed in the main text.

Whereas euploid embryos exhibited a 16% frequency of arrest (95% CI [10%, 25%]), embryos with solely meiotic aneuploidy ("Meiotic only") arrested at a 36% frequency (95% CI [28%, 45%]), while embryos with mitotic aneuploidies ("Meiotic plus mitotic and/or segmental", "Mitotic only", "Mitotic and/or segmental") arrested at much higher frequencies (ranging from 55% to 64%; Figure 3A,B). Moreover, the number of aneuploid chromosomes per embryo was strongly associated with the frequency of arrest, with complex aneuploidies arresting in much higher proportions (Binomial GLMM: average marginal effect [AME] = 0.440, SE = 0.054, $p = 4.04 \times 10^{-16}$; Figure 2C; Figure 3C,D), building upon previous observations from smaller samples (Vega et al., 2014). Interestingly, maternal age was not associated with the frequency of embryo arrest after accounting for aneuploidy status of those embryos (Likelihood ratio test statistic = 0.57, $p = 0.452$), suggesting that the maternal age effect on IVF embryo loss is nearly exclusively mediated by aneuploidy.

Given the strong association between aneuploidy and pregnancy loss, one quantity of fundamental interest is the proportion of embryos derived from euploid zygotes that arrest during preimplantation development, as these compose the initial pool of potentially viable zygotes whose success we seek to maximize. The ability to estimate this parameter has been hindered by the fact that early-arresting embryos are not typically tested in clinical settings. Within our data, the embryos derived from euploid zygotes include any embryo that lacks evidence of meiotic aneuploidy. Embryos in this group arrested at a frequency of 38% (95% CI [31%, 45%]), whereas embryos derived from the remaining aneuploid zygotes arrested at a frequency of 50% (95% CI [44%, 57%]).

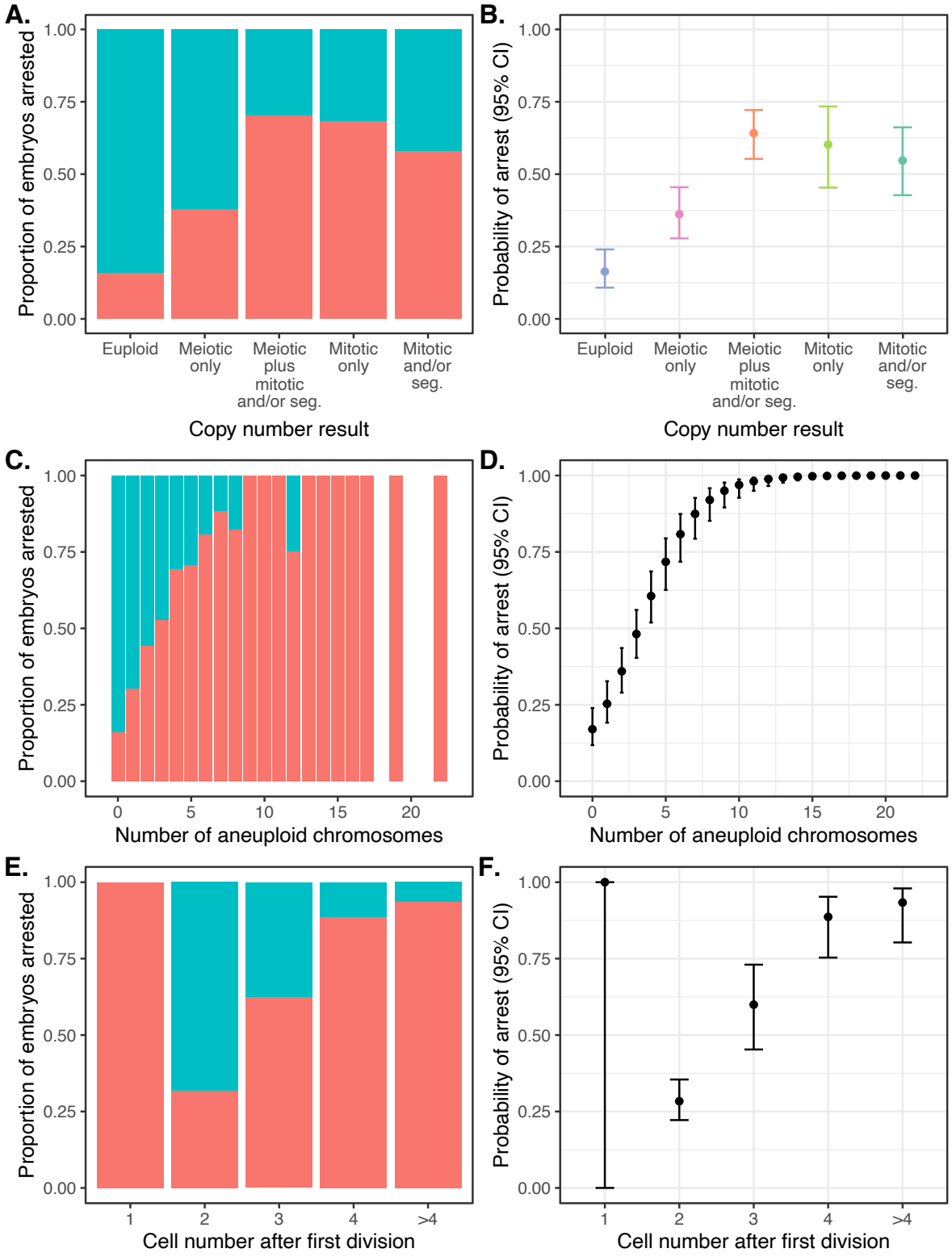

Figure 3. Data (left panels) and statistical modeling (right panels) of the proportion/probability of embryo arrest, stratifying on various patterns of chromosome copy number or cell division. Error bars denote 95% confidence intervals of estimates. **A.** Proportion of embryos arrested (red)

versus unarrested (blue), stratifying on chromosome copy number pattern, as assessed by PGT-A. **B.** Statistical modeling of the data from panel A. **C.** Proportion of embryos arrested (red) versus unarrested (blue), stratifying on the number of aneuploid chromosomes. **D.** Statistical modeling of data from panel C. **E**. Proportion of embryos arrested (red) versus unarrested (blue), stratifying on the number of cells observed after the first mitotic division, where 2 is normal. **F.** Statistical modeling of data from panel E.

Abnormal cell divisions drive lethal mitotic aneuploidies

To gain insight into the relationship between abnormal early cell divisions and mitotic aneuploidies, we used time-lapse imaging (see Methods) to record the first two cell divisions of 843 embryos derived from 2PN zygotes following fertilization. Overall, 219 embryos (26.0%) exhibited an abnormal first division, while an additional 82 embryos (9.7%) exhibited an abnormal second division. The former group includes 85 embryos (10.1%) that underwent tripolar division—the most prevalent form of abnormal division whereby the zygote divided directly into three cells. We observed a strong association between abnormal cell division and putative mitotic aneuploidy, with 51% of abnormally dividing embryos possessing mitotic aneuploidies compared to only 23% of normally dividing embryos (Fisher's Exact Test: OR = 3.41, 95% CI [2.50, 4.67], $p = 6.93 \times 10^{-16}$). No such relationship was observed between abnormal cell division and meiotic aneuploidy (Fisher's Exact Test: OR = 1.20, 95% CI [0.89, 1.62], $p = 0.217$). As further expected, abnormal cell divisions were also strongly associated with embryonic arrest (Fisher's Exact Test: OR = 10.86, 95% CI [7.67, 15.50], $p = 2.72 \times 10^{-50}$; Figure 2D; Figure 3E,F).

Consistent with this interpretation, the probability that embryos derived from euploid zygotes would arrest strongly depended on the outcomes of the first and second cell divisions. Specifically, the probability that an embryo derived from a euploid zygote would arrest was 12% (95% CI [7%, 19%]) when conditioning on normal first and second mitotic divisions compared to 34% (95% CI [27%, 42%]) for aneuploid zygotes. These proportions increase to 75% (95% CI [62%, 84%]) for euploid zygotes as well as 75% (95% CI [66%, 82%]) for aneuploid zygotes when conditioning on abnormal first or second cell divisions.

Notably, even embryos lacking meiotic or mitotic aneuploidies were much more likely to arrest if they experienced an abnormal first or second cell division (Fisher's Exact Test: OR = 19.90, 95% CI [5.59, 90.50], $p = 9.12 \times 10^{-8}$). Though this accounts for only 13 embryos total (compared to 4 euploid embryos that arrested following normal cell divisions), the observed enrichment suggests that the fitness impacts of abnormal cell division are not solely attributable to aneuploidy.

Aneuploidy is strongly associated with blastocyst morphology

Among embryos that persisted to the blastocyst stage, we sought to understand the relationship between various forms of aneuploidy and blastocyst morphology, with the rationale that aneuploidies may compromise the organization and function of the differentiating cell lineages. Blastocysts were graded by assigning an ordinal letter grade (A through D) to the inner cell mass as well as the trophectoderm based on standardized morphological criteria (Table S1; Figure S1; Alpha/ESHRE, 2011). Notably, morphological grading was conducted at the time of embryo culture, prior to obtaining and thereby blind to the PGT-A results. Within our sample, the grades for the inner cell mass and trophectoderm were strongly correlated, consistent with the interpretation that ploidy status and other shared genetic and environmental factors simultaneously impact both cell types (Figure S2). We observed a strong association between aneuploidy status and blastocyst morphology, with aneuploidy rates ranging from 20% to 90% for embryos with the highest to lowest grades. Poor morphology embryos were significantly enriched for aneuploidy (Pearson's Chi-squared Test: X^2 [9, N = 612] = 60.2, $p = 1.23 \times 10^{-9}$; Figure 4). This relationship was consistent for embryos affected by meiotic aneuploidies (Pearson's Chi-squared Test: X^2 [9, N = 612] = 49.4, $p = 1.38 \times 10^{-7}$) as well as those affected with mitotic aneuploidies (Pearson's Chi-squared Test: X^2 [9, N = 612] = 49.3, $p = 1.45 \times 10^{-7}$) when compared to euploid embryos. Additionally, the presence of meiotic and/or mitotic aneuploidies was associated with later timing of blastocyst biopsy, indicating that aneuploidies tend to delay the process of blastocyst formation and expansion (LMM: AME = 0.220, SE = 0.052, $p = 2.4 \times 10^{-5}$; Figure S3).

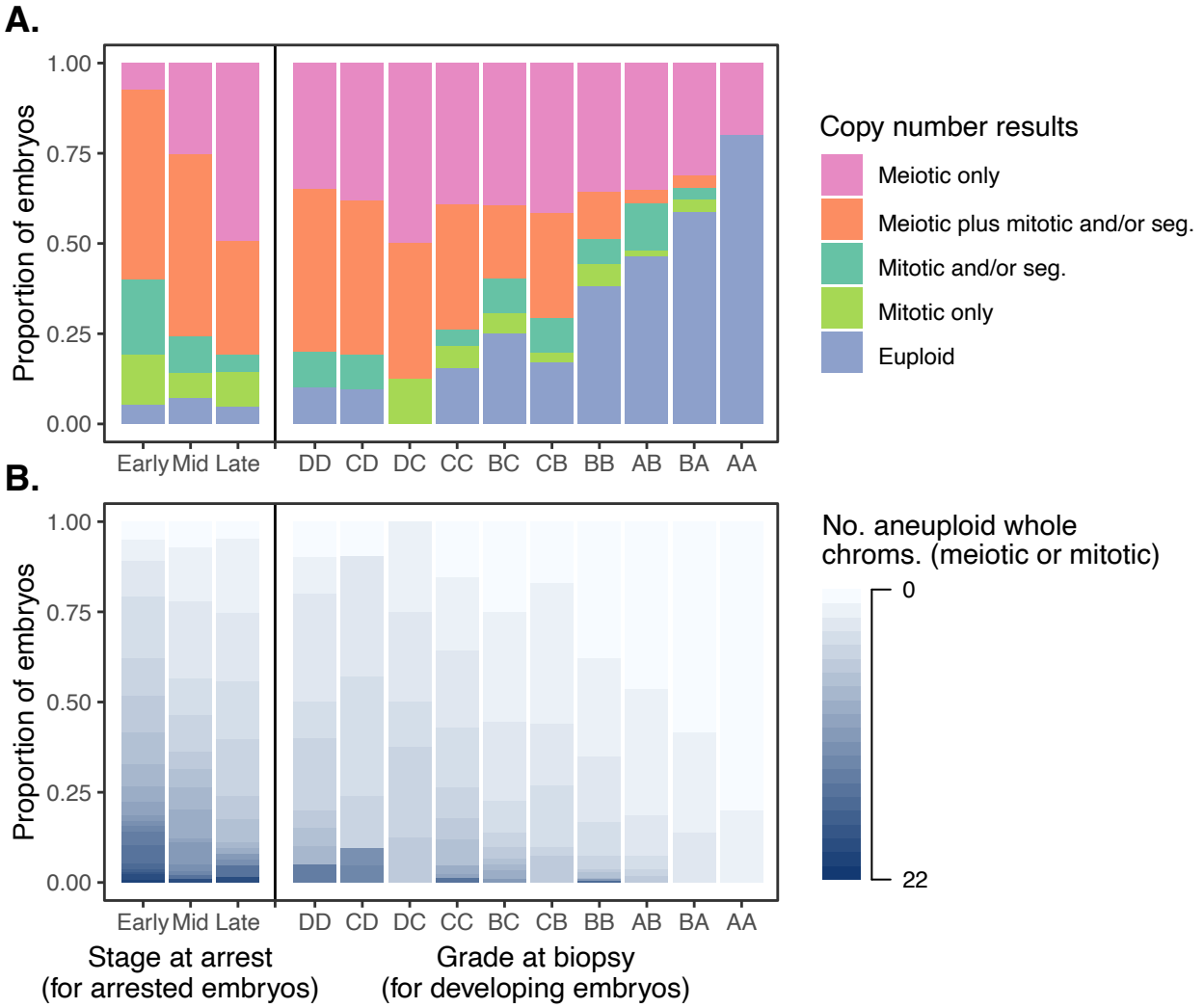

Figure 4. Chromosome copy number results (as assessed via PGT-A) across all tested embryos, stratifying by stage at arrest (see Methods for description of arrested embryos) or morphological grade (for embryos that formed blastocysts). ICM grade is listed first and TE grade is listed second. **A.** Copy number results assigned to categories, as described in Table 1. **B.** Copy number results depicted as counts of aneuploid chromosomes.

Discussion

Here we applied a combination of PGT-A and time-lapse imaging to investigate a large sample of both arrested and developing embryos, irrespective of their morphological grade or any other criteria, toward a more comprehensive view of human preimplantation development. By comparing patterns of putative meiotic, mitotic, whole-

chromosome, and segmental aneuploidies across these samples and stages, we observed that all categories of aneuploidy are enriched in arrested embryos versus developing blastocysts, reflecting their respective contributions to embryonic mortality. The leading cause of IVF embryo arrest appears to be lethal mitotic aneuploidies, which are associated with abnormal first or second mitotic divisions and tend to affect multiple chromosomes simultaneously. Among surviving blastocysts, we further documented a strong association between patterns of aneuploidy and embryo morphology. Together, our results suggest that the transition from the cleavage to blastocyst stage may act as a strong bottleneck wherein numerous aneuploid embryos are eliminated prior to implantation. The timing of this bottleneck is consistent with the increasing reliance on embryonic gene expression as development proceeds through the cleavage, late morula, and early blastocyst stages (Tesarik et al., 1987; Braude et al., 1988; Yan et al., 2013; Blakeley et al., 2015).

Both meiotic and mitotic aneuploidies—though especially the latter—were strongly associated with development arrest. Studies in model systems have demonstrated that aneuploidy tends to compromise cellular fitness (Torres et al., 2007; Stingele at al., 2012). Direct dosage effects of aneuploidy are known to exert proteotoxic and energy stress on cells, increased rates of mutation, and increased rates of chromosome mis-segregation, which may prove lethal during this critical early developmental transition. In particular, excess or depletion of proteins encoded on the aneuploid chromosomes confer stoichiometric imbalances among components of multiprotein complexes, inducing the formation of toxic protein aggregates (Zhu et al, 2018; Brennan et al., 2019; Li et al., 2022). Recent work in aneuploid yeast suggests that these free proteins and protein aggregates increase the solute concentration within cells, causing chronic hypo-osmotic stress that impairs endocytosis (Tsai et al., 2019).

The chromosome instability (CIN) and mitotic aneuploidy observed in the early cleavage-stage IVF human embryos may reflect defects in DNA repair, cell cycle progression, and intracellular signaling as a result of underlying imbalances in cell regulatory networks, metabolic flux, and cellular dynamics (Vanneste et al., 2009; McCoy, 2017; Potapova and Gorbsky, 2017; Tšuiko et al., 2021; Sfakianoudis et al., 2021). Our data demonstrate that these mitotic aneuploidies are prevalent and occur at similar frequencies for embryos derived from zygotes affected or unaffected with prior meiotic aneuploidies, in turn suggesting an intrinsic instability of cleavage-stage development and/or detrimental effects of embryo culture. It has been suggested, for example, that the blastomeres of early cleavage-stage human embryos function autonomously due to the absence of gap junctions and are particularly sensitive to environmental and metabolic perturbations (Brison et al., 2014). By contrast, genome integrity at later stages of development may be more robust, following embryonic

genome activation and the development of a transporting epithelium at the time of compaction.

Human embryo culture media provide only a partial representation (including a protein source, simple salts, energy substrates, and amino acids) of the natural environment to which embryos are exposed *in vivo* (Summers and Biggers, 2003). Consequently stresses are invariably imposed on preimplantation embryos grown in chemically defined media. It is worth recalling that the so-called two-cell block to development first observed in certain strains of mouse embryos when placed in culture, and later shown to be common to all mouse strains, was due to suboptimal culture media (Summers, 2020). Human embryos appear robust and can develop in a wide selection of commercial media, indicating that they compensate and/or adapt to the imposed stresses (Biggers and Summers, 2008), but may ultimately succumb under extreme conditions (Puscheck et al., 2015; Cagnone and Sirard, 2016). The precise environmental tipping points and genetic factors that influence the probability of human embryo arrest merit further investigation.

One caveat of all genetic studies of IVF embryos is the possibility that ovarian stimulation protocols, embryo culture conditions, and blastocyst biopsy could impact the fidelity of chromosome segregation, mosaicism, and/or embryo viability. Highlighting this concern, *in viv*o conceived bovine embryos were shown to exhibit lower rates of mitotic aneuploidy than their IVF counterparts (Tšuiko et al., 2017), though in humans, no such differences were apparent in the fetal or placental lineages, as assessed from tissue biopsies of live-born neonates (Zamani Esteki et al., 2019). Related to these points, previous evidence indicates that the number of cells in the biopsy sample, the fertility clinic, ovarian stimulation protocols, and culture media can influence mosaicism rates (Munné et al., 1997; Munné et al., 2017). Clinical studies assessing the effects of different culture media on rates of aneuploidy, mitotic error and mosaicism are equivocal and like other PGT-A studies have primarily focused on normally developing biopsied blastocysts (Swain, 2019; Swain, 2021). Within our studies, considering those patients that underwent a single euploid blastocyst transfer procedure, the overall average implantation rate and ongoing clinical pregnancy rate/delivery rate across all age groups was 80% and 66%, respectively, providing reassurance about the robustness of the IVF/PGT-A protocols (Gorodeckaja et al., 2019).

In their provocatively titled article, “Where have all the conceptions gone?”, Roberts and Lowe (1975) posited that embryonic mortality is the rule rather than the exception, occurring at a rate of ~80%. Highly cited reviews over subsequent decades offered qualitative support for this conclusion, estimating rates of 60-70% (Chard, 1991; Macklon et al., 2002). Current evidence suggests that a high rate of preclinical losses, not failure of conception, is the main cause of low fecundity of humans, largely driven by

chromosome abnormalities (Brosens et al., 2022). By testing both arrested and unarrested embryos irrespective of morphology, our study supports this conclusion and clarifies the role of meiotic and mitotic aneuploidies in early embryonic mortality. Specifically, we show that severe mitotic errors, which frequently arise due to abnormalities in the initial postzygotic cleavage divisions, are the primary cause of mortality among IVF embryos. Cumulative sustained implantation rates and live birth rates following the transfer of up to three euploid blastocysts have been reported as 95.2% and 92.6%, respectively, *ceteris paribus* (Pirtea et al., 2021). Together, these observations suggest that absent any endometrial or uterine pathology, cumulative pregnancy rates are largely determined by the number of euploid embryos available for transfer, which in turn depends on the proportion of euploid zygotes that proceed through the initial cleavage divisions without error (Reshef et al., 2022). This conclusion motivates crucial future work to identify the factors that modulate risk of mitotic error, toward the goal of expanding fundamental understanding of human fecundity, as well as improving pregnancy outcomes after IVF.

Material and Methods

Study design and informed consent

This report represents a subset of patients that were part of a prospective cohort single center study of patients undergoing IVF with blastocyst vitrification-only and optional PGT-A (VeriSeqPGS, Illumina Inc) between January 2016 to June 2018, previously described by Gorodeckaja et al. (2020).

All procedures performed at the London Women's Clinic, including quality control measures, were approved and licensed by the Human Fertilization and Embryology Authority (HFEA) in the United Kingdom in accordance with all relevant regulations and legislation. Details of ovarian stimulation protocols and laboratory procedures have been described in Gorodeckaja et al. (2019). No new procedures, protocols, or randomization were used in the program. Consequently, the study does not constitute human subjects research and accordingly approval from an Institutional Review Board was not required. Consenting of patients was carried out in accordance with the Code of Ethics of the World Medical Association (Helsinki Declaration). All patients undergoing PGT-A were consented by a qualified clinician and licensed genetic counselor. Written informed consent for genetic testing and follow up analysis, including the anonymous use of embryology data for statistical evaluation and research was reviewed, signed and witnessed. The Homewood Institutional Review Board of Johns Hopkins University

determined that the analysis of these data does not qualify as federally regulated human subjects research (HIRB00011431).

Time-lapse image analysis and blastocyst grading

Time-lapse incubation was used to maintain an uninterrupted embryo culture environment. Embryo development was monitored continuously up to seven days post insemination, or until blastocyst formation and expansion, if earlier, by time lapse imaging and software analysis (Geri® Connect, GeneaBiomedx, Sydney, Australia). Blastocysts were evaluated by assigning a letter grade (A through D) to the inner cell mass (ICM) and trophectoderm (TE) based on standardized morphological criteria (Table S1; Figure S1; Alpha/ESHRE, 2011). Grading of top quality blastocyst was more restrictive, whereas very poor quality blastocysts were given a D grade based on very few cells in the ICM and TE and evidence of cellular degeneration. Time lapse videos of all embryos were annotated daily using the manufacturer's software and documentation in the patient's laboratory record. Assessment included several time points in the development of each embryo: (1) formation of pronuclei, (2) first cleavage division, (3) second cleavage division, (5) cell compaction, (6) cavitation, (7) expanded blastocyst, or (8) embryo arrest image prior to processing embryos for PGT-A. The division pattern was recorded for each embryo as per Ottolini et al. (2017) and based on the number of cells after each division to indicate either normal (e.g., 1-2-4 cells) or abnormal (e.g., 1-3-6 cells) cleavage patterns (Rubio et al., 2012; Ciray et al., 2014; Kalatova et al., 2015; Coticchio et al., 2021).

Preparation of whole arrested embryos

Embryos that showed no evidence of further development by either cell count or compaction and failed to develop to the expanded blastocyst stage by day 7 or showing signs of degeneration were considered arrested in development if no change was seen by time-lapse analysis for at least the preceding 24 hours. Arrested embryos were scored as either early, mid, or late as follows: early arrest, 1 cell through 6-10 cells to cover post-fertilization through embryonic genome activation; mid arrest, >10 cells to pre-compaction; and late arrest, evidence of compaction through early blastocoel formation. The zona pellucida of each arrested embryo was first thinned by brief exposure to acidified Tyrode's solution (Origio, USA) and removed by gentle pipetting, ensuring the integrity of the entire intact cell mass for subsequent genetic analysis. All selected arrested embryos were then prepared for genetic analysis. DNA isolation, sample preparation, and whole genome amplification (WGA) was carried out as previously described (Gorodeckaja et al., 2019).

Data processing, statistical analysis, and visualization

Raw sequencing data were processed and visualized with Illumina Bluefuse Multi. Intra-sample mosaicism is diagnostic of mitotic error and is expected to produce copy number results intermediate between those expected of uniform meiotic aneuploidy and disomy. We defined the range for putative mitotic (i.e., mosaic) aneuploidy at 30-70% of the expected copy number change for uniform trisomies or monosomies, in accordance with previous work (Ottolini et al., 2017; Handyside et al, 2021). To allow for technical variability, values below that threshold were considered as putative normal disomies, whereas values above that threshold were considered as putative meiotic aneuploidies. While it is likely that a small proportion of embryos were mis-classified with this approach, we emphasize that the same criteria were applied across the entire sample, thereby ensuring robustness of comparisons between embryos at different stages or with different developmental outcomes. Sex chromosome aneuploidies and segmental aneuploidies were not stratified into meiotic and mitotic aneuploidy categories, because the probability of mis-classification is much higher for these groups. In the case of sex chromosome aneuploidies, the baseline copy number expectations depend on the assumed sex of the embryo, which itself must be inferred. In the case of segmental aneuploidies, the smaller affected regions result in a lower ratio of signal to noise in normalized read depth. Moreover, individual genomic bins may encompass CNV breakpoints, causing the erroneous detection of intermediate copy number segments.

All statistical analysis was conducted in R (version 4.1.2). Figures were produced using the ‘ggplot2’ (Wickham, 2011) and ‘ggsankey’ (https://github.com/davidsjoberg/ggsankey) packages.

The relationships between probability of embryo arrest and various predictor variables (Figure 3) were modeled using binomial generalized linear mixed effects models (GLMMs), implemented with the ‘GLMMadaptive’ package (https://drizopoulos.github.io/GLMMadaptive/). Data were randomly down-sampled to achieve proportionate sample sizes of arrested embryos to blastocysts tested by PGT-A. Patient identifier was included as a random effect in all models, thereby addressing the non-independence among sets of embryos sampled from the same patient. Specifically, in the first model, the response variable was a binary indicator denoting whether or not the embryo arrested, while the copy number result category was the sole categorical predictor variable. The second model used the same response variable, but the total number of whole aneuploid autosomes (meiotic or mitotic) was the sole numeric predictor variable. The third model again used the same response variable, but the number of cells after the first mitotic division was included as the sole categorical predictor variable, where the possible categories were 1, 2, 3, 4, and >4. In all cases, coefficient estimates and 95% confidence intervals were converted from the logit to

probability scale to facilitate interpretation. As coefficient estimates from GLMMs are conditional on the level of the random effect (i.e., patient), we report the average marginal effect (AME), which averages over all levels of the random effect.

The relationship between aneuploidy and day of blastocyst biopsy was modeled using a linear mixed effects model (LMM), implemented with the 'lme4' package (Bates et al., 2015), with AMEs computed using the 'margins' package (https://thomasleeper.com/margins/).

Data and software availability

De-identified chromosome-level aneuploidy calls and time lapse results for all tested embryos are available on Zenodo (doi: 10.5281/zenodo.6789485), along with de-identified metadata for the corresponding patients and cycles. Code necessary for reproducing all analyses and figures is available at https://github.com/mccoy-lab/embryo_arrest and archived on Zenodo (doi: 10.5281/zenodo.6789485).

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Disclosure statement

RCM is co-inventor on a provisional patent application by Johns Hopkins University related to inferring the origins of aneuploidies from PGT-A data. AHH, MCS, CO, AM and KA report no conflicts of interest.

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